Efficacy in Reduction of Lung Lesions of a Toxin Expressing Whole-cell Vaccine Against Multiple Serovars of *Actinobacillus Pleuropneumoniae* in Growing Pigs

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Abstract

Background: *Actinobacillus pleuropneumoniae* is a major economically significant bacterial respiratory pathogen of pigs, and vaccine use is considered an integral control method to prevent disease. The objective of this multi-study analysis was to evaluate the serovar independent efficacy in growing pigs of the C-vaccine (Coglapix®, Ceva, France), which comprises whole cells of *A. pleuropneumoniae* serovars 1 and 2 expressing ApxI, ApxII and ApxIII toxins. Efficacy was based on protection against lung lesions, since there is good correlation between the severity/extension of lung lesions and losses induced by pleuropneumonia. Vaccine efficacy was determined against challenge with the most common serovars (1, 2, 4, 5, 6, 7, 9/11 and 13) of *A. pleuropneumoniae* in a total of 13 studies of the same design and reproducibility was validated.

Results: Protection against homologous serovars 1 and 2 significantly reduced lung lesion scores (LLS) compared to the positive controls: $p = 0.00007$ and $p = 0.00124$, respectively. The protection against heterologous serovars 4, 5, 6, 7, 9/11, and 13 also significantly reduced LLS: range $p = 2.9e-10$ to $p = 0.00953$. Reproducibility between challenge studies was excellent with the estimated random effect of study (the fraction of the total variation attributed by differences between studies) being only 2.6%, 2.2% and 4.8% for three serovar 2, two serovar 9/11 and serovar 4 challenge studies, respectively. An outlier was the 35% of variation attributable to trial between the two serovar 6 challenges, possibly explained by a *Streptococcus* spp. outbreak.

Conclusions: A highly significant serovar independent reduction of pathological lung lesions by the C-vaccine was demonstrated for all the serovars tested (1, 2, 4, 5, 6, 7, 9/11 and 13). High levels of protection with similar significance-values were obtained for both homologous and heterologous serovar challenge. To our knowledge the aerosol chamber challenge concept based on accurate individual pig dosing and a standardized biologically weighted lung lesion scoring is the first to be validated statistically as reproducible and reliable. The C-vaccine was demonstrated to be a good candidate to fulfil the demands in the field for an *A. pleuropneumoniae*-vaccine i.e., high protective capability against disease caused by multiple serovars.

Background

Actinobacillus pleuropneumoniae, the aetiological agent of swine pleuropneumonia, is responsible for substantial morbidity and mortality causing substantial economic losses in the global pork industry. Pleuropneumonia in its peracute and acute forms is mainly characterised by severely affected well-being and increased mortality. Pigs that survive infection, including after antimicrobial treatment, are likely to develop chronic disease characterised by reduced activity and appetite. Acute-peracute clinical signs are generally clear and distinct plus chronic pleurisy is easily diagnosed via slaughterhouse investigations^{1,2,3,4}. Pigs diagnosed with subacute pleuropneumonia show milder, less distinct clinical signs and lower fatality. While subclinical pleuropneumonia may involve pathological pneumonic lesions, no clinical signs may be apparent^{3,5}. How accurate the distinction between the subclinical and subacute forms are in the

individual case will rely highly on the frequency and quality of on-farm health monitoring. Some subclinical cases may very well be missed, for example, in farms where animals are co-infected with *Mycoplasma hyopneumoniae*. The subclinical form of *A. pleuropneumoniae* negatively affects growth rate and feed efficiency⁵ due to chronic lung lesions, like pleurisy and adherence together with a fibrino-haemorrhagic and necrotizing pleuropneumonia, as commonly seen at the abattoir⁶.

Estimations on the economic burden of this disease is mainly based on the occurrence of acute outbreaks characterized by high mortality and medical costs; few reports on production efficacy parameters like average daily weight gain (ADG) and/or feed conversion ratio (FCR), and even less have measured losses due to subacute and/or subclinical pleuropneumonia³. An analysis of five publications on 14 trials, found mean improvements due to antimicrobial intervention in ADG of 33.6% and FCR of 25.5%, with high variations⁷. In a controlled long-term field study including a total of 33063 pigs, C-vaccinated (were compared to non-vaccinated for the control of acute pleuropneumonia, mortality was reduced by 28% ($p = 0.011$). However, the improvements on feed efficiency by 9.36 kg feed per pig produced ($p = 0.023$), and ADG by 40 per day or pen-efficacy by 6.3% ($p < 0.001$) will mainly be attributable to subclinical-subacute pleuropneumonia occurring earlier than the observed recurrent outbreaks⁸.

The appearance of lung lesions, like pleurisy, at the abattoir, is often associated with *A. pleuropneumoniae* (OR = 8.75)^{9,10}, and can be linked to decreased ADG, increased FCR, prolonged stay in finishing compartment, and reduced carcass weight^{11,12}. Pleurisy present at slaughter reduced lifetime weight gain by 1.25 kg on post trimming carcass weight, equal to 1.66 kg of live weight, in average. Also, a 10%-increase of affected lung tissue was correlated of to a reduction of 3.3-4,6% on ADG¹³.

Lung lesion scoring is considered highly relevant for estimating severity and losses of respiratory disease, such as caused by *A. pleuropneumoniae*, at the farm level^{14,15,16,17}. To investigate pleuropneumonia in all its possible manifestations, pathological evaluation of lung lesions appears to be the least biased method. Performing this evaluation close to pneumonic infection would seem to reveal the most accurate validation of the degree of pleuropneumonic impact on the individual pig.

Lung lesion scoring as the endpoint of measuring on *A. pleuropneumoniae* induced disease is widely accepted^{18,19,20,21,22,23,24,25,26,27,28,29,30}. A dose-response relation exists^{31,18,22,23}. Depending on the challenge dose of the individual serovar one can achieve any stage of disease from absolute mortality^{32,33,19,20,21,22}, even in bacterin vaccinated pigs²¹, to subacute^{18,24} even to subclinical pleuropneumonia^{18,21,24}. However, due to high variation in disease inside *A. pleuropneumoniae* challenged groups^{18,21,22,23,24,27,28,29,30,31} a calculation of a dose-response will always produce a variation in lung lesions; least when mortality is the outcome. Here the lung lesions are severe and extensive affect 80–100% of the parenchyma^{19,20,25,27,30,31,32,33}.

A. pleuropneumoniae is endemic world-wide and swine farms are often infected with more than one serovar. In some countries 80–90% of farms are estimated to be seropositive for *A. pleuropneumoniae*, with 1–6 out of 7 serogroups present at the same time: 93% having ≥ 2 and 80% having ≥ 3

simultaneous Ap-serogroups present ^{34, 35}. The prevalence of serovars varies between countries, regions of countries, and by year of investigation ^{35, 36, 37, 38, 39, 40}.

So far nineteen *A. pleuropneumoniae* serovars have been classified worldwide ⁴¹. However, as the difference between serovar 9 and 11 is only one amino acid in the complete CPS loci and they have identical toxin profiles (ApXI, ApXII), these two serovars can be considered as one: serovar 9/11 ⁴². Strains belonging to different serovars are highly different in virulence and in some cases different strains of the same serovars can express different pathogenicity features; usually due to different Apx toxin profiles investigation ^{35, 36, 37, 38, 43}. More extrinsic factors like general stress ^{3, 43, 44}, poor air quality and climatic control, particularly high ambient temperature variations over the day, are associated with increased severity of pathological lesions caused by *A. pleuropneumoniae*, even in the case of what are considered low virulent *A. pleuropneumoniae* strains^{3, 6}.

A. pleuropneumoniae has several virulence factors, some are well described, and several are under investigation. The three exotoxins: ApXI-III and lipopolysaccharide (LPS) have been proven responsible for lung lesions, but at the same time are both immunogenic and can induce protective immunity ^{1, 3, 46}. Many *A. pleuropneumoniae* virulence factors have been described ⁴⁶ including outer membrane proteins (OMPs), some of which are immunogenic and therefore potential vaccine candidates ⁴⁷.

The variation in virulence between *A. pleuropneumoniae* serovars is mainly determined by the production of one or two of the ApXI-III toxins. These exotoxins providing nutrients for further growth and activity via lysis of the nearby cells in the lung tissue including neutrophils and macrophages ^{48, 49}. LPS are both adhesion factors, allowing for colonisation and the production of exotoxins, and at the same time enhancing the cytotoxic effects of ApXI-III ^{50, 51, 52, 53}.

Several commercial vaccines are available which differ in their composition and can be appointed into one of three *A. pleuropneumoniae* vaccine categories: 1) killed *A. pleuropneumoniae* whole-cell components only (bacterins); 2) subunit vaccines containing ApXI-III toxins only; and 3) a combination of these ⁴⁷. With distinct differences in efficiency, they all reduce clinical signs, but none can fully prevent infection and colonization ⁵⁴. Antibodies against ApXI-III are responsible for the serovar-independent protection against lung lesions^{3, 6, 46, 47}. Due to limited cross protection between the serovars, bacterin vaccines lack efficacy compared to ApXI-III combined bacterin vaccines; pure toxoids vaccines lack in general protective capacity due to lack of LPS and other cell wall components ^{55, 56, 57, 58}. *A. pleuropneumoniae* vaccine group 3 above is quite heterogenous, with a wide variety in composition, from a subunit vaccine containing the ApXI-III and only one of the cell wall OMPs, to the vaccine evaluated in this study based on whole-cell components of two serovars, together expressing all three of the ApXI-III toxins, and to vaccines containing whole-cell components of several *A. pleuropneumoniae* serovars together with some exotoxins.

A combination of the three exotoxins, ApXI-III with LPS, and likely more of the abundant cell-wall based antigens ^{56, 58}, induces a strong and specific cell mediated immune response that can confer serovar

independent protection³. This is an effective design for an efficacious serovar-independent vaccine, feasible for *A. pleuropneumoniae* prophylaxis to: increase animal well-being, reduce antimicrobial use, and reduce losses due to pleuropneumonia in all its manifestations at any *A. pleuropneumoniae*-endemic farm at any time^{3, 6, 47}.

The objective of this study was to evaluate the efficacy of a vaccine comprising whole cells of *A. pleuropneumoniae* serovars 1 and 2 which in combination express ApxI, ApxII and ApxIII to protect against pleuropneumonic lungs lesions following challenge with multiple prominent serovars of *A. pleuropneumoniae* in growing pigs.

Materials & Methods

Data from thirteen studies each including one of the eight *A. pleuropneumoniae* serovars 1, 2, 4, 5, 6, 7, 9/11 and 13 performed over the period of 2011 to 2020 were available (Table 1). Where data on multiple studies with the same serovar were available weighted lung lesion score (LLS) of the vaccinated group of pigs (Vac) versus the non-vaccinated pigs in the positive control group (Pos) were pooled and analysed while taking the potential effect of study into account (Table 1). Also, variance between studies on the same serovars were analysed to estimate quality of repeatability.

Table 1

List of *A. pleuropneumoniae* challenge trials used in in this analysis. The two studies performed by Ceva R&D are intranasal dosed challenges (IN). the 11 studies performed by Ceva SSIU are all aerosol chamber dosed challenges.

Serovar	Origin	Strain ID	Dose CFU/pig	Test Group	Pigs / group	Year of study	Pig breed	Official approval ID
1	Denmark	<i>App. St1 ch BS5689</i>	4 x 10 ⁸ IN	Vac	8	2012	Hungaro- Seghers	<i>BA01/2005- 1/2010</i>
				Vac	8			
				Pos	7			
2	Hungary	<i>App. St2 ch 2008/3 + 2</i>	1 x 10 ⁶	Vac	11	2015	Topigs- Norsvin x PIC	<i>BA01/2005 és 1/2010- 1/2012</i>
				Pos	11			
			1 x 10 ⁷	Vac	9	2017	Danbred	<i>BA02/2000- 43/2017</i>
				Pos	10			
			1 x 10 ⁸	Vac	10	2014	Hungaro- Seghers	<i>BA01/2005 és 1/2010- 1/2012</i>
				Pos	10			
4	Spain	<i>App.90993</i>	1 x 10 ⁸	Vac	10	2018	Danbred	<i>BA02/2000- 43/2017</i>
				Pos	9			
			1 x 10 ⁸	Vac	20	2020	Danbred	<i>BA02/2000- 43/2017</i>
				Pos	20			
5	Italy	<i>App St.5 13/ITA</i>	1 x 10 ⁶	Vac	10	2011	Hungaro- Seghers	<i>BA01/2005- 1/2010</i>
				Pos	10			
6	Denmark	<i>App. J.no. 101059 + 2SP</i>	1 x 10 ⁸	Vac	10	2018	Danbred	<i>BA02/2000- 43/2017</i>
				Pos	9			
			1 x 10 ⁸	Vac	18	2019	Danbred	<i>BA02/2000- 43/2017</i>
				Pos	14			
7	Hungary	<i>App. St7 ch CH.G-I/7- 7/12</i>	2.8 x 10 ⁸ IN	Vac	18	2012	Hungaro- Seghers	<i>BA01/2005- 1/2010</i>
				Pos	14			
9/11	Hungary	<i>App. St.9 ch (B- 2011)</i>	1 x 10 ⁶	Vac	11	2012	Hungaro- Seghers	<i>BA01/2005- 1/2010</i>
				Vac	10			

Serovar	Origin	Strain ID	Dose CFU/pig	Test Group	Pigs / group	Year of study	Pig breed	Official approval ID
13	Spain	<i>App.99865 + 1</i>	1×10^7	Pos	10	2014	Hungaro- Seghers	<i>BA01/2005 és 1/2010- 1/2012</i>
				Vac	10			
				Pos	10			
				Vac	20			
13	Spain	<i>App.99865 + 1</i>	1×10^7	Pos	20	2020	Danbred	<i>BA02/2000- 43/2017</i>
				Vac	20			

The trial designs were all the same (see Trial design) and in accordance with the European Pharmacopeia⁵⁹. All trials were performed by Ceva Research and Development (R&D) Department or Ceva Scientific Support and Innovation Unit (SSIU) in Hungary.

The vaccine

The vaccine tested was Coglapix® (Ceva Santé Animale, France) hereafter referred to as C-vaccine. The C-vaccine is based on whole cells of *A. pleuropneumoniae* serovars 1 and 2 expressing ApxI, ApxII and ApxIII. Over the span of years, the vaccine composition and quality control has not changed. Apart from the cross-protective Apx-toxins, this vaccine contains all principal cell wall structures of *A. pleuropneumoniae* bacteria in undetermined quantities which contribute to *A. pleuropneumoniae* protective immune response: LPS, OMP's and the several other cell wall components.

Serotyping of the challenge strains

Strains belonging to different serovars were isolated from clinical cases of swine pleuropneumonia and serotyped using hyper immune sera by indirect haemagglutination as described previously⁶⁰.

Serotyping of all *A. pleuropneumoniae* strains was confirmed in a multiplex-PCR based on capsular loci carried out as described by Bossé and colleagues⁶¹.

Calibration and preparation of the challenge strains

Strains were assessed for their ability of growth in liquid culture in a Tryptic soy broth supplemented with yeast extract and nicotinamide adenine dinucleotide solution in shake-flasks rotated at 180 rpm and kept at 37°C. Their growth curve was analysed using sampling at pre-determined sampling points and subsequent optical density (OD) measuring at different wavelengths using a standard laboratory photometer. At each sampling point, the cultures were subjected to colony forming unit (CFU) counts using standard bacteriological techniques. The OD and CFU values were then aligned and the strain-specific, optimal wavelengths were determined. After this initial procedure, in each case when a challenge

trial was performed, the strain used was prepared in shake flasks under regular OD monitoring and stopped when reaching the desired live titre based on the OD-CFU calibration curve.

Aerosol dosing technique

Challenge strains were propagated and used for the test when 10^9 CFU/ml concentration was reached. The *A. pleuropneumoniae* stock was diluted in sterile PBS to achieve the optimal required 10^6 , 10^7 or 10^8 CFU/animal treatment dosage as shown in Table 1. Actual calculations were made at the test site, using the following parameters to introduce 1 dose/animal during the aerosol treatment to the chamber:

- Pig body weight ⁶² and volume
- Number of pigs placed in the chamber for one run (6, 8 or 10)
- Volume of chamber.
- Volume of liquid, turned to aerosol by the ultrasonic nebulizer in 10 minutes (usually 100–150 ml, depending on air temperature and humidity)

The pigs were evenly distributed and secured in the chamber by partition fences; aerosol was created by an ultrasonic humidifier and uniformly dispersed by internal ventilation. After 10 minutes of treatment, the pigs were kept in the chamber for an additional 2 minutes with the nebulizer switched off, to allow complete uptake of the aerosol droplets (fresh air was provided during this time to allow normal breathing). Before the first run, the chamber was moisturized with the nebulizer to prevent aerosol loss caused by adherence to the dry surfaces; before each run piglets introduced to the chamber are given a couple of minutes of ease to ensure normal respiration before the doors are closed and the challenge is initiated. This aerosol chamber concept is developed by Palya and Kiss, and inspired by previous work on aerosol chambers^{18, 32, 63}.

Intranasal (IN) challenge

Production of the challenge strain and calibration of the challenge dose was as described above. The cultures were prepared in $10\times$ concentration of the desired challenge titre and diluted in sterile PBS to reach the working concentration. Each animal received 5 ml of challenge dose into each nostril using intranasal cannulas; the exact individual animal dose is shown in Table 1.

Pre-trial determination of challenge dose

Prior to using the strains in vaccine challenge trials, challenge dose calibration studies were performed. In these trials, three groups of 10 non-vaccinated *A. pleuropneumoniae*-negative pigs were challenged with doses of 10^6 , 10^7 or 10^8 CFUs, monitored daily for clinical signs and euthanized one week later. Mortality and LLS were evaluated to select the optimal challenge dose to be used: a mortality of 20–30 % in the non-vaccinated control group.

For *A. pleuropneumoniae* 2 and *A. pleuropneumoniae* 9/11 the pleuropneumonic impact of different concentrations of challenge dose were investigated via LLS to evaluate the *A. pleuropneumoniae*

protective capabilities of the vaccine for different challenge loads.

Inclusion criteria

Pigs of either sex and of different breeds changing over time (Table 1) were recruited from farms free of *A. pleuropneumoniae*, *Mycoplasma hyopneumoniae*, toxin-positive *Pasteurella multocida* (progressive atrophic rhinitis), porcine reproductive and respiratory syndrome virus, Aujeszky's disease virus, classical swine fever virus and African swine fever virus based on regular PCR and/or serology tests performed either by government or private labs. Also, the animals had no previous clinical history of infection by *Streptococcus suis* and *Glaesserella parasuis*.

Any animal selected for inclusion in *A. pleuropneumoniae* challenge trials had to be negative in the ApxIV ELISA (IDEXX APP-ApxIV Ab) test when serum sampled at 5–6 weeks of age, confirming that they were negative for both *A. pleuropneumoniae* infection and colostral *A. pleuropneumoniae* antibodies.

Trial design

The challenge trials were performed using the same overall study design. The only difference was the use of an aerosol chamber challenge model by SSIU and an intra-nasal (IN) application by R&D (Table 1). Detailed methods are given below.

Pigs at the age of 7–8 weeks, were randomly assigned to either non-vaccinated or vaccinated groups and housed indoors, with controlled temperature and ventilation. Each pig of the vaccine group (Vac) received the first 2 ml dose of the C-vaccine by intramuscular injection (D0). Three weeks later, D21, the pigs of the Vac group received a second 2 ml dose intramuscular of the same vaccine; the controls received no treatment. Pigs were randomised according to bodyweight and staff responsible for the daily care and monitoring of the pigs were not involved in vaccination and unaware of which pigs belonged to which test-groups.

At D42 all pigs individually received pre-determined equal doses of the relevant virulent *A. pleuropneumoniae* strains either by application in an aerosol chamber, or by the IN route, as described above.

At D49, one-week post-challenge, the trials were terminated. All live pigs were humanely euthanized and pathoanatomically evaluated to establish the individual lung lobe lesions to calculate the individual LLS. Persons performing the pathoanatomical evaluation were not involved in vaccination and unaware of which pigs belonged to which test-groups.

For *A. pleuropneumoniae* 2, three studies, for *A. pleuropneumoniae* 4, 6 and 9/11, two studies, and for the remaining *A. pleuropneumoniae* serovars, one study were included in the analyses.

Post-mortem evaluation of weighted lung lesion score (LLS)

In the vaccination-challenge trials, all animals euthanised on day 7 post-challenge, D49, were subjected to necropsy to investigate the pathological changes due to actinobacillosis. Evaluation of the post-mortem

lesions in the lungs and on the pleura were performed blind and in accordance with a previously described scoring system ¹⁵. All seven lobes of the lung of each pig in trial were examined and each lobe scored on prevalence of pathological lesions of pneumonia and/or pleuritis (pleuropneumonia). Score valuing was according to the size of the affected area: absence = score 0, 1–20% = score 1, 21–40% = score 2, 41–60% = score 3, 61–80% = score 4, and 81–100% = score 5 ¹⁵.

Weighting factors were applied on all individual lung-lobe scorings according to the relative size of each lobe in the lung of each pig: right-cranial = 0.07, right-middle = 0.15, right-caudal = 0.35, accessory-lobe = 0.05, left-cranial = 0.04, left-middle = 0.09, and left caudal = 0.25 ⁶³. Pigs that had died during the week following challenge and before termination of the trial were given the maximum lung lesion score of 5. This way each pig lung ended up with a total LLS of 0–5; the more lesions the higher the score.

Statistical analyses

The effect of vaccine on LLS was analysed using linear (mixed) models separately for each *A. pleuropneumoniae* serovar. If more than one study was available, a random effect of study to account for the possible clustering of effects within a study was included. To assess the importance of the between study variation, the intraclass correlation coefficient (ICC) was calculated as the proportion of the total variation attributed to the random effect. For the outcome (LLS), a limit of detection (LOD) was defined as half the minimum observed LLS. The LOD was added to all LLS before it was log transformed to improve the underlying assumption about normal distribution of data. All analyses were done in R ⁶⁵, using the lme4 ⁶⁶ package for statistical analyses of mixed effects models with the lmerTest ⁶⁷ package for testing of significant effects.

Results

The protection of the C-vaccine against the homologous *A. pleuropneumoniae* serovars 1 and 2 strains was demonstrated with highly significant reductions of LLS compared to the positive controls: $p = 0.00007$ and $p = 0.00124$ respectively (Table 2). The protection of the C-vaccine against the heterologous *A. pleuropneumoniae* serovars 4, 5, 6, 7, 9/11, and 13 was demonstrated with equally highly significant reductions in LLS: $p = 2.9e-10$ to $p = 0.00953$ (Table 2). The negative controls in all studies stayed *A. pleuropneumoniae* negative and without any LLS to be observed.

Table 2

Sample size, mean Lung Lesion Score (LLS) and standard deviation (SD) for vaccine groups and positive controls for *A. pleuropneumoniae* serovars (A.p.) 1, 2, 4, 5, 6, 7, and 9/11. The p-value is for the test of a difference between vaccine and positive controls within each *A. pleuropneumoniae* type. Significance is considered when the p value < 0.05

A.P. serovar	Vaccine			Positive Control			
	n	LLS	SD(LLS)	n	LLS	SD(LLS)	P-value ¹
A.p. 1	16	0.23	0.31	7	1.96	1.14	0.00007
A.p. 2	30	0.75	1.22	31	2.11	2.05	0.00124
A.p. 4	30	0.65	0.41	29	1.46	1.25	0.00044
A.p. 5	10	0.18	0.54	10	1.18	1.61	0.00953
A.p. 6	28	0.71	0.60	23	1.56	1.13	0.00195
A.p. 7	17	0.04	0.10	17	1.17	1.15	2.9e-10
A.p. 9/11	31	2.26	1.89	20	3.84	1.82	0.00663
A.p. 13	20	1.03	1.44	20	2.66	1.93	0.00319
¹ All tests of significance were done on the log(LLS) to improve the underlying assumptions of the analysis							

Some variation in mean LLS was observed between *A. pleuropneumoniae* serovars, supporting the decision to analyse each *A. pleuropneumoniae* serovar separately (Table 2). For *A. pleuropneumoniae* 2, the estimated random effect of study was (variance = 0.071) and the residual variance was 1.860, which implies an ICC = $0.05 / (0.05 + 1.88) = 0.026$, i.e., only 2.6% of the total variation was due to differences between studies. For *A. pleuropneumoniae* 9/11 the ICC = $0.02 / (0.02 + 0.87) = 0.022$, i.e., 2.2%, and for *A. pleuropneumoniae* 4 ICC = $0.05 / (0.052 + 0.99) = 0.048$, i.e., 4.8%. This suggests a standardized set-up, where the effect of study essentially can be ignored in the analyses. However, for *A. pleuropneumoniae* 6, the ICC = $0.54 / (0.54 + 1.01) = 0.35$, i.e., 35%, suggesting that there were marked differences between these two studies.

Discussion

The concept of combining the ApxI, ApxII and ApxIII^{3, 6, 46, 47} with cellular components of *A. pleuropneumoniae*^{55, 57} has been demonstrated to result in a highly effective serovar-independent vaccine that reduces lung lesions and mortality, and improves production performance. The use of such an efficacious vaccine will increase animal well-being, and reduce both antimicrobial use and economic losses due to endemic pleuropneumonia^{3, 6, 47}.

To our knowledge, this is the most exhaustive testing on any *A. pleuropneumoniae*-vaccine, whether approved to be serovar independent or not by the relevant authorities. In these studies, we have analysed the efficacy of the C-vaccine in protecting against lung lesions of eight different virulent field strains of *A. pleuropneumoniae* (1, 2, 4, 5, 6, 7, 9/11 and 13); six heterologous and two homologous with the serovars on which the vaccine is based. We found a significant reduction in LLS for the vaccinated groups compared to the positive controls. This implies that the vaccine is capable of inducing serovar-independent protection and is a valuable characteristic for optimizing the control of *A. pleuropneumoniae*-related pig health problems.

The majority of the studies we carried out used an aerosol chamber. When considering challenge models, for most investigators, the choice is between IN or aerosol chamber challenge (AC). IN has an inherited accuracy in applied dose but is labour intensive and comparatively more expensive. In addition, dependent on pig handling and dose application (e.g., sedation/non-sedation), IN is potentially more stressful which can increase respiratory rate, hence respiratory volume and can affect the planned dose. With AC, skilful pig handling can ensure acceptance of the animals to the chamber and less stress. Our results indicate that reproducible protection studies can be performed using aerosol chambers with, to our knowledge, the Ceva Phylaxia aerosol chamber *A. pleuropneumoniae* challenge concept, being the only one validated for reproducibility using the intraclass correlation coefficient (ICC). Where determined, reproducibility between challenge studies was high, hence the outcome-of-trial data can be considered of high reliability, reflecting accurate individual challenge dose calculations, and basing the lung lesion scoring on standardized methodology¹⁵ adapted to the biological appearance of the lung⁶⁴ achieving standardized, reproducible, weighted lung lesion score (LLS).

The variations attributable to differences between studies are very low: 2.6%, 2.2% and 4.8% for three *A. pleuropneumoniae* 2, two *A. pleuropneumoniae* 9/11 and two *A. pleuropneumoniae* 4 challenge studies, respectively. An outlier is the 35% of variation attributable to trial between the two *A. pleuropneumoniae* 6 challenges. An explanation could be that pleuritis was generally observed in a larger proportion of the animals in the 2019-study. Bacteriology demonstrated the presence of *Streptococcus* spp. in these samples. Significant improvement in LLS compared to the control group was still observed in this trial alone, and even more so when analysed together with the 2018-study. That infection with other pathogens, e.g., *Bordetella pertussis*, can affect lesion score in *A. pleuropneumoniae* challenged animals has been documented by others³⁰.

Others have used aerosol chambers to challenge pigs with *A. pleuropneumoniae*^{18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33}. In these sixteen publications five common and important serovars were investigated: seven on serovar 1^{19, 20, 21, 22, 31, 32, 33}, four on serovar 7^{26, 28, 29, 30}, two on serovar 2^{23, 24}, one on serovar 5 only²⁵, one on serovars 2, 5 and 6¹⁸, and one on serovars 2 and 9²⁷. Serovars 2, 5 and 6 were concluded as being of moderate to high virulence¹⁸, but this was based on small numbers of animals being investigated, and the result with serovar 5 can be considered surprising given that this is normally considered as of high virulence⁴⁸. Serovar 7 was considered as moderately virulent³⁰. Based on very high doses in identical trial designs, serovars 1 and 5 appear comparable in virulence measured on mortality

only ^{20, 25}. When comparing dosage and outcome empirically across the heterogeneous trial designs, serovar 1 stands out as the most virulent closely followed by serovars 5 and 9, placing serovars 2, 6 and 7 as moderate to highly virulent. However, most of these studies, like ours, were not designed to reveal differences in virulence, rather dosing was aimed at obtaining similar disease severity distributions in the positive control groups to enable assessment of vaccine protection. Nonetheless, our data indicates broad agreement with the literature in that serovars 1, 5, 9/11 are the most virulent, serovars 2 and 13 of slightly lesser virulence, and serovars 4, 6 and 7 as moderate-to-highly virulent. It should be noted that the serovar 2 isolate we used was from Europe which expresses ApxII and ApxIII which is of higher virulence than serovars 2 isolates from North America which typically only express ApxII ³⁷. A definite rating of the virulence of different serovars by AC would require fully standardised extensive head-to-head trials to be carried out in a reproducible challenge model similar to that presented and validated in this publication.

In this study we have used the LLS to assess vaccine efficacy after aerosol challenge to *A. pleuropneumoniae*. In the 16 aerosol challenge papers previously reported, 28 tests groups can be identified, with five reporting mortality and describing lung lesions in general pathological terms ^{19, 31, 32, 33}, three are using in-house models including other than the lungs ^{18, 21, 22}, two calculate percentage of lung tissue affected ^{19, 25}. Only seven score lung lesions with the standard scheme of Hannan and colleagues ^{15, 23, 24, 26, 27, 28, 29, 30}. None of them used a weighted lung lesion score which takes into account the size of the individual lung lobes for optimal comparison between pigs and groups.

Also, the days from challenge to scoring varies substantially between the 28 groups: twelve are in the interval of 15 to 22 days and only focused on chronic lesions ^{19, 21, 22, 23, 26, 27, 28, 29, 30, 33}, three are intermediate from 12–14 days ^{19, 33}, nine are focused on acute, subacute and subclinical lesions in the interval of 5–7 days ^{19, 20, 23, 24, 25, 27, 29, 31, 32}, and one was assessed only at 24 hours ¹⁹; in three publications comparisons were made between groups where dead animals were not part of the analyses are not included here ^{22, 27, 29}. Finally, numbers are predominantly small with only 5/28 groups using 10 animals or above ^{21, 22, 30}, another five used 8 pigs ^{19, 23, 25, 26, 28}, and the majority using less pigs in a test group ^{18, 19, 20, 22, 23, 24, 27, 29, 30, 31, 32, 33}. Thus, the variation in both dosing and assessment methodology severely complicates comparisons with other reported aerosol challenge studies. Further comparative studies would best be undertaken in a highly standardised model with reproducible methodology, such as that we report here. Also, further research in methods to validate *A. pleuropneumoniae* induced pleuropneumonic losses in general is key, but of particular interest for further evaluation of the subclinical/subacute forms ^{3, 18, 21, 24}. In a world of reducing antimicrobial use, the ability to perform exact cost-benefit analyses on different *A. pleuropneumoniae* control strategies are already of great importance, and involvement of systematic LLS is likely to be an integral component of such schemes ¹⁴.

Conclusions

The C-vaccine was highly effective, providing serovar-independent highly significant reductions of LLS in multiple challenges with different *A. pleuropneumoniae* serovars 1, 2, 4, 5, 6, 7, 9/11 and 13.

To our knowledge the aerosol chamber challenge concept is the only one validated for reproducibility, basing challenge on individual pig challenge doses and weighted lung lesions scores for the most accurate biological evaluation of disease and vaccine protection against *A. pleuropneumoniae* disease.

Declarations

Ethics approval and consent to participate

The trial designs were all the same and in accordance with the European Pharmacopeia ⁵⁹. All trials were performed by Ceva Research and Development (R&D) Department or Ceva Scientific Support and Innovation Unit (SSIU) in Hungary.

All studies followed local law and regulations. Authorization was provided by the Government Office of Baranya County Food Chain-Safety and Animal Health Department, Hungary. Individual study approval ID noted (Table 1).

The studies were conducted in accordance with the provision Directive 2010/63/EU (still in force), Hungarian act XXVII/1998 (still in force, with regular updates in content) and the Hungarian Ministerial Decree No. 243/1998, replaced the 15.04.2013 by the 40/2013. (II. 14.) Hungarian Governmental Decree, prepared according to the Directive 2010/63/EU.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PM generated this multi-analysis, gathered the data and acted as primary, corresponding author. NT analysed all data in the capacity of being a skilled independent statistician experienced in multi and meta-

analyses. IK together with VP developed the Ceva aerosol chamber concept and are responsible for the production of the challenge data of the SSIU, plus reviewed and contributed importantly in writing. HS reviewed and contributed importantly in writing. MT is responsible for the production of the challenge data of the R&D, plus reviewed and contributed importantly in writing.

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